VETERINARSKI ARHIV 85 (4), 395-406, 2015

Preparation of microspheres containing leptospiral antigen using biodegradable alginate polymers

Fatemeh Inanlou[1], Pejvak Khaki[2]*, Naser Mohammadpour[3], Soheila Moradi Bidhendi[2], Hossein Zolfagharian[3], Yung-Fu Chang[4], Mohammad R. Bazrafshan[5], and Forough Saraei[1]

[1]*Department of Microbiology, Islamic Azad University, Karaj Branch, Karaj, Iran*

[2]*Leptospira Reference Laboratory, Department of Microbiology, Razi Vaccine and Sera Research Institute, Karaj, Iran*

[3]*Department of Venom and Human Sera, Razi Vaccine and Sera Research Institute, Karaj, Iran*

[4]*Department of Population, Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA*

[5]*Alborz University of Medical Sciences, Karaj, Iran*

INANLOU, F., P. KHAKI, N. MOHAMMADPOUR, S. MORADI BIDHENDI, H. ZOLFAGHARIAN, Y. F. CHANG, M. R. BAZRAFSHAN, F. SARAEI: Preparation of microspheres containing leptospiral antigen using biodegradable alginate polymers. Vet. arhiv 85, 395-406, 2015.

ABSTRACT

Leptospirosis is a worldwide zoonosis caused by pathogenic *Leptospira* spp. Although the currently available whole cell leptospiral vaccines can induce protection against Leptospirosis, further study for a new generation of vaccine that can stimulate long-term immunity is needed. Biodegradable microspheres as antigen delivery systems have been extensively investigated for decades, especially those based on hydrophilic polymers, such as alginate and chitosan, which have excellent biocompatibility, non-toxicity and biodegradability. The aim of this study was to prepare and characterize alginate microspheres as an antigen delivery system for immunization against leptospirosis. Alginate microspheres containing Leptospiral antigen (LA) were prepared by an emulsification method and characterized for shape, size distribution, loading efficiency (LE), loading capacity (LC) and release profile. The effects of some parameters (such as concentration of alginate and emulsifiers and stirring rate) on microspheres characteristics were investigated. The optimal condition parameters for the preparation of LA loaded alginate microspheres were estimated. The optimum concentrations were obtained for alginate and emulsifiers, 3.5 % (w/v), span 80 (0.2 % w/v) and tween 80 (3.75 % w/v), respectively. Moreover, appropriate homogenizing rate was obtained at 500 rpm. Our results showed the mean

*Corresponding author:

Dr. Pejvak Khaki, Razi Vaccine and Serum Research Institute, Beheshti Ave, Hessarak, Postal Code: 3197619751, Karaj, Iran, Phone: +98 263 457 0038_2250; Fax: +98 263 445 2194; E-mail: P.Khaki@rvsri.ac.ir

ISSN 0372-5480
Printed in Croatia

particle size of the microspheres as 200 μm, loading efficiency 97 % and loading capacity 8 %. A suitable release profile was observed for in vitro release test of LA from alginate microspheres over an extended period of time (192 hours). These results make the alginate microspheres particularly interesting for an LA delivery system.

Key words: *Leptospira* spp., leptospiral antigen, alginate, microspheres, antigen delivery, immunization

Introduction

Leptospirosis is a serious worldwide zoonosis caused by pathogenic *Leptospira* spp, and has also been identified as a re-emerging infectious disease, particularly in tropical and subtropical regions (YAN et al., 2009). Transmission to humans occurs via contaminated water or soils, through the urine of infected animals. Moreover, the overall disease burden is underestimated, since leptospirosis is a significant cause of undifferentiated fever and is frequently not recognized. Barriers to addressing this problem have been the lack of adequate diagnostic tests and effective control measures. The presently available vaccines impart only short-term immunity, mediated by opsonizing antibodies, and fail to provide cross-protection against the large number of pathogenic serovars (ANDRE-FONTAINE et al., 2003). Although cell-mediated immunity is mostly directed against intracellular pathogens, several studies have demonstrated that protective immunity against *Leptospira* is correlated with Th1 responses, characterized by CD4+ and γδ T cell production of IFN-γ (KLIMPEL et al., 2003). An ideal vaccine against *leptospira* infection would therefore activate both humoral and cell-mediated immune responses (FAISAL et al., 2009a).

Although a number of potent adjuvants are available, adverse reactions due to toxicity have limited their use in vaccine formulations. Aluminum hydroxide (Alum) is the only adjuvant approved for human use but primarily induces humoral immune responses and only limited cell-mediated immunity. In addition, Alum is not effective for induction of mucosal immunity and can cause allergic reactions in some cases (GUPTA, 1998). The particulate antigen delivery systems (such as microspheres, liposomes, virosomes, etc.) are very potent adjuvants and have been widely used against various infectious diseases (BEHBOUDI et al., 1995).

Microencapsulation is a process in which the cells are retained within an encapsulating matrix or membrane. Microencapsulation of antigens has been investigated for improving their immunogenicity. This process provides a long-term depot for the antigen and leads to inducing effective and long lasting immunity (KRASAEKOOPT et al., 2004).

Several particulate antigens, containing live or dead microorganisms have been proved to be effective immunogens, by using biodegradable polymers such as poly (lactic-co-glycolic acid) (PLGA), poly-L-Lactid (PLLA) and alginate (FLICK-SMITH et al., 2002; YEH et al., 2002; FLORINDO et al., 2008). Immunization studies against leptospirosis via PLGA microspheres and liposomes have revealed that these particles are promising adjuvants for use with vaccines against leptospirosis (FAISAL et al., 2009a,b). The most widely used

encapsulating material is alginate, a linear heteropolysaccharide of D-mannuronic and L-guluronic acid, which is extracted from various species of algae (KRASAEKOOPT et al., 2004). Recently, alginate microspheres have been used in various immunization studies (DOBAKHTI et al., 2006; TAFAGHODI et al., 2007; ARENAS-GAMBOA et al., 2008). There are hardly any data about the use of alginate microspheres as an adjuvant for use with vaccines against leptospirosis. Therefore in the present study, we investigated the optimum conditions for the preparation of alginate microspheres containing leptospiral antigens as a delivery system for immunization against leptospirosis.

Materials and methods

Materials. Low viscosity sodium alginate (Sigma Chemical Co., USA), calcium chloride, Span 80, Tween80 and iso-octane (Merck, Germany) were employed for particle preparation. All other reagents used were of at least analytical grade.

Leptospira culture and preparation of antigen. Leptospira interrogans serovars Grippotyphosa (RTCC 2808), Sejroe hardjo (RTCC 2821) and Canicola (RTCC 2805) were obtained from the *Leptospira* Reference Laboratory, Razi Vaccine and Serum Research Institute, Karj, Iran. The bacteria were inoculated into Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco Laboratories) at 28° C, for 7-10 days. Bacterial growth was monitored using dark-field microscopy. The bacteria were centrifuged at 17000 rpm for 20 min and the cell pellets were resuspended in phosphate buffered saline (PBS, pH 7.2), the cells were inactivated by formalin 0.5 % (v/v) and the viability of bacteria were checked and then they were stored at 4 ºC for 24 h. The cell suspensions contained 10 to 20 mg of cells (in terms of dry weight) per milliliter.

Preparation of particles. Alginate microspheres containing leptospiral antigen (LA) were prepared by the modified emulsification method (JIN et al., 2009). Briefly, LA was suspended in 10 mL 2-5 % (w/v) alginate solution and mixed with 20 mL iso-octane containing 0.2-2 % (w/v) Span 80. The mixture was emulsified for 3 min in a homogenizer (Heidolph, Germany) at 500-1000 rpm, followed by the addition of 1.5 mL 3.75-30 % (w/v) tween 80 aqueous solution as the second emulsifier, to achieve a suitable hydrophilic-lipophilic balance (HLB) value, and the mixture was further stirred at the same speed for 3 min. Then, 8 mL 8 % (w/v) calcium chloride solution was added dropwise. This cross-linking process lasted for 3 min. After the mixture was stirred for another 3 min, the alginate microspheres were collected by centrifugation, washed twice with deionized water and then the microspheres were suspended in 20 mL of poly-L-lysine 0.1 % w/w. After 10 min homogenization, the alginate microspheres collected by centrifugation and then washed with deionized water and finally dried in a vacuum desiccator.

Particle characterization. The morphology of LA loaded microspheres was observed using optical microscopy (BX51, Olympus Corporation, Japan). The detailed morphology of these microspheres was studied by scanning electron microscopy (SEM) (Oxford, UK). Microspheres for SEM were mounted on metal stubs previously covered with double-side adhesive, and coated with gold in vacuum conditions. Particle size (volume mean diameter) and size distribution of the microspheres were determined using a particle size analyzer (Malvern, UK).

Loading efficiency (LE) and loading capacity (LC) of LA in alginate microspheres. The LE was calculated as the ratio between the mass of encapsulated agent in the recovered particles and the total mass of bacteria added during particle production. The LC was estimated as the ratio between the mass of the encapsulated agent in the recovered particles and the total mass of microspheres during particle production (JIN et al., 2009). After dissolving 80 g of dry particles with 5 mL of 0.75 M sodium citrate aqueous solution, overnight (LEMOINE et al., 1998), the samples were centrifuged at 10,000 rpm for 10 min and supernatant was neutralized by 0.1 M sodium hydroxide. Sample absorbance was determined at 585 nm and compared to a previously prepared standard curve. For each batch of microspheres the LE and LC were determined in triplicate.

In vitro antigenicity of LA. A critical point in developing a carrier system for antigens is the preservation of their native antigenicity. During alginate microsphere preparation, LA was exposed to potentially harsh conditions, such as contact with surfactants and organic solvents. This may result in alteration of the structural stability and decrease of antigenicity of the antigen. Therefore, the in vitro antigenicity of LA was measured by enzyme-linked immunosorbent assay (ELISA) (FAISAL et al., 2009a). Each sample was examined at different dilutions against a linear scale showing the responses of control standard samples with different dilutions. The in vitro antigenicity of LA was evaluated by the ratio of ELISA response and protein concentration (ELISA/protein).

In vitro release studies. The in vitro release studies of alginate microspheres containing LA were carried out in PBS (pH 7.2). Accurately weighed amounts of LA loaded alginate microspheres were put into eppendorf tubes (about 20 tubes) so that each tube contained 1 mg of the microspheres and 1 mLof PBS. The eppendorf tubes were then incubated at 37 °C under continuous shaking for 1 week. At various time intervals, the supernatant (250 µL) was drawn after centrifugation (13000 rpm for 10 min) and replaced with fresh medium. The LA released into the supernatant was quantified by Bradford protein assay method. The in vitro release studies were performed in triplicates.

Results

The effects of emulsion preparation conditions on particle characteristics. The influence of the preparation conditions, such as the concentration of alginate solution,

the concentration of emulsifiers and the homogenizing rate, on the characteristics of the microspheres are shown in Tables 1-3. The results showed that the most influential parameter for the characteristics of the microspheres was the homogenizing rate, followed by the concentration of alginate solution and the concentration of emulsifiers in turn. The optimal parameters of the process were as follows: the concentrations of alginate solution and emulsifiers were 3.5 % (w/v), span 80 (0.2 % w/v) and tween 80 (3.75 % w/v), respectively and the homogenizing rate was 500 rpm (Tables 1-3).

Table 1. Effect of sodium alginate concentration on microsphere formation

Alginate concentration (% w/v)	Yield of microspheres (mg)	Microsphere formation
1.5	152 ± 2	Ag
2.5	221 ± 1.73	Ag
3.5	272 ± 2.08	P
5	401.6 ± 2.88	D

Ag: aggregation, P: proper microsphere, D: deformed microsphere

Table 2. Effect of emulsifier concentration on microsphere characteristics

Span 80 (% w/v)	Tween 80 (% w/v)	Yield of microspheres (mg)	Emulsion stability and particle size
0.1	2	229.66 ± 2.51	NS and P
0.2	3.75	272 ± 1.75	S and P
0.5	15	284.65 ± 3.51	S and IMP
1	30	300 ± 2.21	S and IMP

NS: non-stable emulsion, S: stable emulsion, P: proper microsphere, IMP: improper microsphere

Table 3. Effect of homogenizing rate on microsphere characteristics

Homogenizing rate (rpm)	Yield of microspheres (mg)	Emulsion stability and particle size
500	274 ± 1.53	S and P
700	252.66 ± 2.07	S and IMP
1000	242 ± 2.64	S and IMP

S: stable emulsion, P: proper microsphere, IMP: improper microsphere

Morphology, size and particle size distribution. The obtained alginate microspheres and LA loaded alginate microspheres were characterized by SEM and a particle size analyzer. Two typical scanning electron microphotographs of blank microspheres and LA loaded microspheres are presented in Fig. 1. According to the figure, the alginate microspheres were almost spherical (Fig. 1A), and alginate microspheres containing LA were spherical without any aggregations (Fig. 1B). The average size of the alginate

microspheres was about 189 µm and the mean size of LA loaded alginate microspheres was about 200 µm, which was larger than the blank microspheres. The size distribution of alginate microspheres and LA loaded alginate microspheres are presented in Fig. 2, which indicates that well dispersed alginate microspheres were prepared.

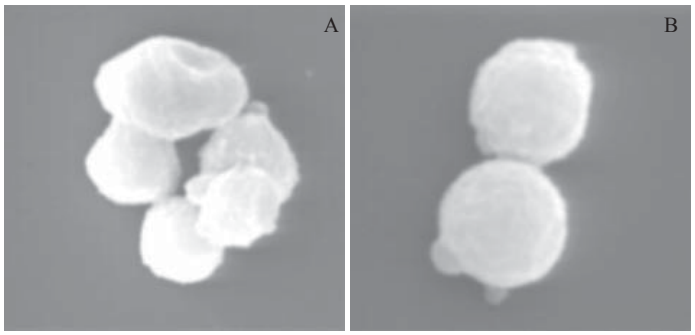

Fig. 1. SEM images of alginate microspheres before loading (A) and after loading with antigen (B)

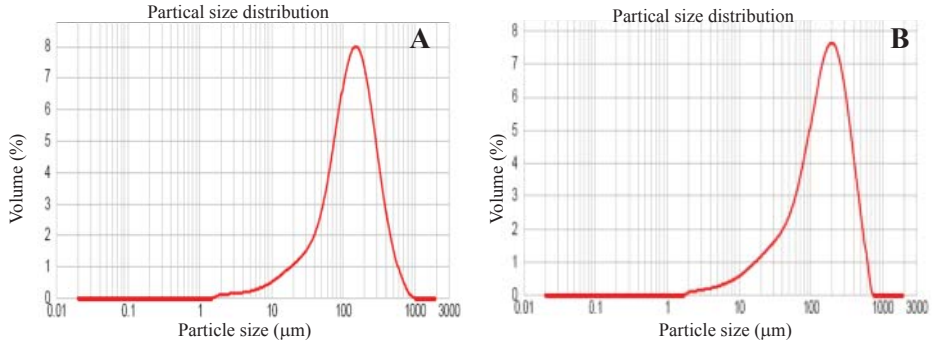

Fig. 2. Size distribution profile of alginate microspheres before loading (A) and after loading with antigen (B)

LE and LC of LA in alginate microspheres. To assess the ability of the alginate microspheres to incorporate the inactive bacteria, the effects of the initial LA content on LE and LC were evaluated. Fig. 3 shows the effect of LA concentration on the LE and LC of alginate microspheres. As shown in Fig. 3, on increasing LA concentration, the LE and LC were increased. The LA with 20 mg/mL (10^{10} cell/mL) concentration led to maximum LE (97.41 %) and LC (8 %). The alginate microspheres with a concentration of 20 mg/mL antigen were adapted to in vitro release.

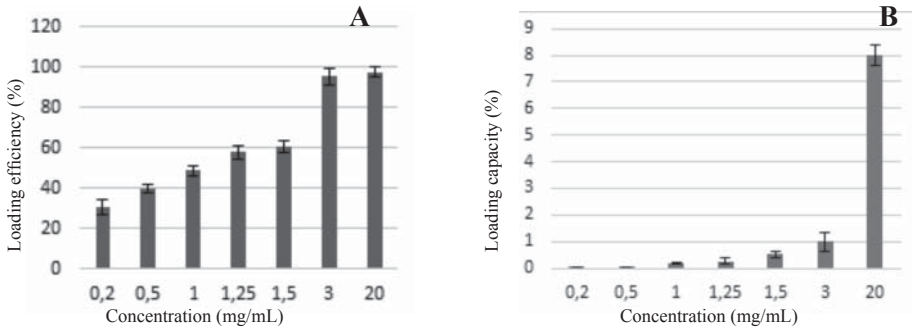

Fig. 3. Effects of different LA concentrations on loading efficiency (A) and loading capacity (B) of sodium alginate microspheres

In vitro antigenicity. The antigenicity of LA encapsulated in microspheres was evaluated by ELISA/protein ratio determination. Almost identical results were observed for the native LA and the LA released from the alginate microsphere (data not shown). There was no significant antigenicity loss (10 % ± 2) induced by the encapsulation and subsequent release of LA from the alginate microspheres.

In vitro release. The in vitro release of LA from the alginate microspheres was investigated in phosphate buffer solution (pH 7.2) at 37 °C. Slow release of LA from alginate microspheres occurred in the first 8 h, subsequently release was faster up to 48 h and over the next 192 h with an approximately constant rate, and about 64 % of LA were released in 5 days (Fig. 4).

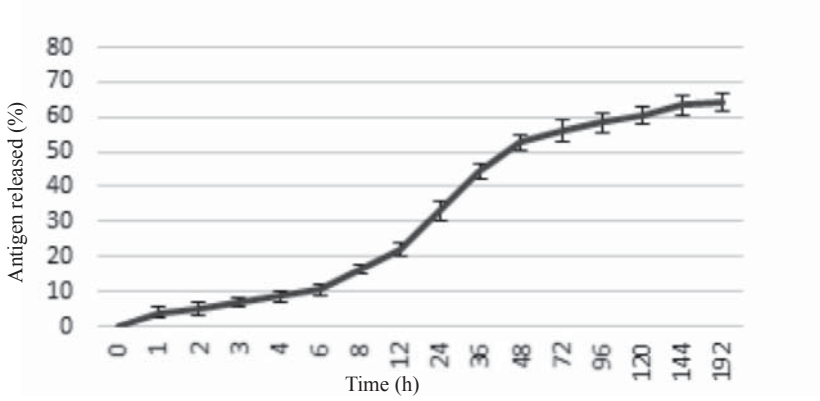

Fig. 4. *In vitro* profile of LA from alginate microspheres

Discussion

Leptospirosis, a zoonotic disease, has been recognized as an important emerging infectious disease over the last 10 years. Prevention of leptospirosis without vaccination is impractical and difficult to achieve. Currently available vaccines consist of killed whole cell bacterins, which are used widely in animals, but less so in humans (ADLER and MOCTEZUMA, 2009). Existing licensed vaccines have several drawbacks, including requiring multiple doses to induce protective efficacy, and repeated annual revaccination to retain immunity. Therefore, safe and efficacious immunization systems are needed to overcome these disadvantages. Hydrophilic microspheres have received much attention for delivery of therapeutic peptide, protein and antigen by intravenous, oral, and mucosal administration. Alginate has been widely used in pharmaceutical research as a carrier for drug and antigen delivery.

Faisal et al. showed that the variable region of recombinant *Leptospira* immunoglobulin like protein A (LigAvar), incorporated into conventional liposomes and PLGA microspheres, produced robust immune responses that induced significant protection against virulent *L. interrogans* serovar Pomona challenge in hamsters (FAISAL et al., 2009a). This group, in another study, prepared novel liposomes from total polar lipids of non-pathogenic *Leptospira biflexa* serovar Patoc (designated leptosomes) and evaluated their vaccine adjuvant potential with novel protective antigens (Lp0607, Lp1118 and Lp1454) of *L. interrogans* serovar Pomona in a hamster model. Their results demonstrated that leptosome is a better adjuvant than conventional liposomes, as revealed by enhanced long term antibody response, lymphocyte proliferation and significant enhancement of both Th1 (IFN-γ) and Th2 (IL-4 and IL-10) cytokines (FAISAL et al., 2009b).

In the present study we tried for the first time to produce biodegradable alginate microspheres for loading leptospiral antigens, and to evaluate their potential as delivery systems.

The results obtained from the study indicated that use of sodium alginate with concentrations lower than 3.5 % w/v led to the formation of low mechanical resistance microspheres due to diluting the polymeric solution. Such microspheres were small in size but during the production or drying procedure, due to high external energy, indicated coalescence and aggregation. In contrast, the samples with concentrations higher than 3.5 % w/v sodium alginate were mostly deformed in shape and large in size. The probable reason was the high viscosity of the polymeric solution. Thereby, stirrer could not homogenize the viscose aqueous phase in the organic phase and produced deformed microspheres. These results were in accordance with previous reports (LEMOINE et al., 1998; RODRIGUES et al., 2006; TAFAGHODI et al., 2006) but they are contrary to results reported by CHO et al. (1998). Of course it may be mentioned that the high homogenizing rate used by them may have caused the adverse results in comparison with other groups.

Our study revealed that appropriate microspheres were fabricated with 0.2 % w/v span 80 and 3.75 % w/v tween 80. Similar to results reported by others (WAN et al., 1994; ZHENG et al., 2004; JIN et al., 2009), in lower concentrations of emulsifiers the obtained emulsion was not stable and in higher concentrations of emulsifiers the size of the microspheres decreased too much as a result of the reduction of the surface tension of the dispersed phase droplets.

Whereas one of the most influential parameters for the characteristics of the microspheres was the homogenizing rate, the use of a suitable stirring rate may be significant. On the basis of on evaluations in this study, stable and proper microspheres were made with a higher stirring rate (500 rpm), similar to results previously reported by other researchers (CHO et al., 1998; RODRIGUES et al., 2006).

The effects of LA concentration on LE and LC were investigated and our results showed that by increasing the LA concentration, the LE and LC were increased. The LA at a 20 mg/mLconcentration led to maximum and considerable LE (97.41 %) and LC (8 %). Our results were similar to the majority of other experiences with alginate microspheres (WAN et al., 1992; KIM et al., 2002; SANGEETHA et al., 2007).

In the current study, to estimate the particle size distribution and mean diameter of the alginate microspheres, normal particle size distributions before and after loading with LA were determined. The mean diameter of microspheres after loading with LA was increased. The enhancement of the microspheres' diameter may be caused by electrostatic bonds between polymeric chains and antigen proteinic chains on the surface of the microspheres. SEM photographs of LA loaded microspheres showed that alginate microspheres were almost spherical without any aggregation, as also reported by other studies (TAFAGHODI et al., 2006; JIN et al., 2009).

Alginate matrixes have been proven to be useful for the slow release of several potential therapeutic proteins, and several studies have demonstrated the usefulness of these systems (TAFAGHODI et al., 2007). Similarly in our study, the in vitro release profile of LA loaded microspheres showed the long release time and slow release rate of LA result from the fact that LA macromolecules were bound to the microspheres by strong interaction. Additionally, after augmentation of poly-L-lysine, the electrostatic interaction between the positively charged -NH3+ of poly-L-lysine and negatively charged -COO- of alginate redounded to increase the stability of the microspheres. The presence of poly -L-lysine layer could reduce the diffusion of LA from the microspheres and prolong the release time of the LA. Subsequently, due to degradation and erosion of the matrix, the LA might be released from the alginate microspheres in an extended profile. The results obtained by the present study were confirmed by LEMOINE et al. 1998, and ANAL et al. 2003.

Conclusion

The properties of prepared microspheres containing leptospiral antigens were strongly dependent on both sodium alginate and emulsifier concentrations, and the stirring rate of the emulsion was a critical factor for the preparation of suitable microspheres. The optimum concentrations of alginate and emulsifiers led to the formation of the desirable microspheres and consequently high loading efficiency, loading capacity and sustain release profile. Therefore it is suggested that the alginate microspheres containing leptospiral antigens could probably be used as an adjuvant for vaccination against leptospirosis.

Acknowledgements

This study was supported by a grant from the Razi Vaccine and Serum Research Institute Karaj, Iran.

References

ADLER, B., A. D. MOCTEZUMA (2009): Leptospira and leptospirosis. Vet. Microbiol. 4, 4382-4392.

ANAL, A. K., D. BHOPATKAR, S. TOKURA, H. TAMURA, W. F. STEVENS (2003): Chitosan-alginate multilayer beads for gastric passage and controlled intestinal release of protein. Drug. Deliv. Indian. Pharm. 29, 713-724.

ANDRE-FONTAINE, G., C. BRANGER, A. W. GRAY, H. L. KLAASEN (2003): Comparison of the efficacy of three commercial bacterins in preventing canine leptospirosis. Vet. Rec. 153, 165-169.

ARENAS-GAMBOA, A. M., T. A. FICHT, M. M. KAHL-McDONAGH, A. RICE, A. C. FCHIT (2008): Immunization with a single dose of a microencapsulated *Brucella melitensis* mutant, enhances protection against wild-type challenge. Infect. Immun. 76, 2448-2455.

BEHBOUDI, S., B. MOREIN, B. RONNBERG (1995): Isolation and quantification of *Quillaja saponaria* Molina saponins and lipids in iscom-matrix and iscoms. Vaccine 13, 1690-1696.

CHO, N. H., S. Y. SEONG, K. H. CHUN, Y. H. KIM, I. C. KWON, B. Y. AHN, S. Y. JEONG (1998): Novel mucosal immunization with polysaccharide-protein conjugates entrapped in alginate microspheres. Control. Rel. 53, 215-224.

DOBAKHTI, F., S. AJDARY, M. TAGHIKHANI, S. H. RAFIEI, K. H. BAYATI, M. RAFIEE-TEHRANI (2006): Immune response following oral immunization with BCG encapsulated in alginate microspheres. Iran. J. Immunol. 3, 114-120.

FAISAL , S. M., W. YAN, S. P. MCDONOUGH, Y. F. CHANG (2009a): Leptospira immunoglobulin-like protein A variable region (LigAvar) incorporated in liposomes and PLGA microspheres produces a robust immune response correlating to protective immunity. Vaccine 27, 378-387.

FAISAL, S. M., W. YAN, S. P. McDONOUGH, C. F. CHANG, M. J. PAN, Y. F. CHANG (2009b): Leptosome-entrapped leptospiral antigens conferred significant higher levels of protection than those entrapped with PC-liposomes in a hamster model. Vaccine. 27, 6537-6545.

FLICK-SMITH, H. C., J. E. EYLES, R. HEBDON, E. L. WATERS, R. J. BEEDHAM, T. J. STAGG, J. MILLER, H. O. ALPAR, L. W. BAILLIE, E. D. WILLIAMSON (2002): Mucosal or parenteral administration of microsphere-associated *Bacillus anthracis* protective antigen protects against anthrax infection in mice. Infect. Immun. 70, 2022-2028.

FLORINDO, H. F., S. PANDIT, L. M. GONCALVES, H. O. ALPAR, A. J. ALMEIDA (2008): *Streptococcus equi* antigens adsorbed onto surface modified poly-Ɛ-caprolactone microspheres induce humoral and cellular specific immune responses. Vaccine 26, 4168-4177.

GUPTA, R. K. (1998): Aluminum compounds as vaccine adjuvants. Adv. Drug. Deliv. 32, 155-172.

JIN, M., Y. ZHENG, Q. HU (2009): Preparation and characterization of bovine serum albumin alginate/chitosan microspheres for oral administration. Pharm. Sci. 4, 215-220.

KIM, B., T. BOWERSOCK, P. GRIEBEL, A. KIDANE, L. A. BABIUK, M. SANCHEZ, S. ATTAH-POKU, R. S. KAUSHIK, G. K. MUTWIRI (2002): Mucosal immune responses following oral immunization with rotavirus antigens encapsulated in alginate microspheres. Control. Rel. 85, 191-202.

KLIMPEL, G. R., M. A. MATTHIAS, J. M. VINETZ (2003): *Leptospira interrogans* activation of human peripheral blood mononuclear cells: preferential expansion of TCR gamma delta+ T cells vs TCR alpha beta+ T cells. Immunol. 171, 1447-1455.

KRASAEKOOPT, W., B. BHANDARI, H. DEETH (2004): The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria. Int. Dairy. J. 14, 737-743.

LEMOINE, D., F. WAUTERS, S. BOUCHEND'HOMME, V. PREAT (1998): Preparation and characterization of alginate microspheres containing a model antigen. Int. J. Pharm. 176, 9-19.

RODRIGUES, A. P., D. HIRSCH, H. C. FIGUEIREDO, P. V. LOGATO, A. M. MORAES (2006): Production and characterisation of alginate microparticles incorporating *Aeromonas hydrophila* designed for fish oral vaccination. Process Biochem. 41, 638-643.

SANGEETHA, S., D. NAGASAMY-VENKATESH, R. ADHIYAMAN, K. SANTHI, B. SURESH (2007): Formulation of sodium alginate nanospheres containing amphotericin B for the treatment of systemic candidiasis. Trop. J. Pharm. Res. 6, 653-659.

TAFAGHODI, M., S. A. SAJADI-TABASI, M. R. JAAFARI (2006): Formulation and characterization and release studies of alginate microsphere encapsulated with tetanus toxoid. Biomat. Sci. Polymer. Ed. 17, 909-924.

TAFAGHODI, M., S. A. SAJADI-TABASI, M. PAYAN (2007): Alginate microsphere as a delivery system and adjuvant for autoclaved *Leishmania major* and Quillaja saponin: preparation and characterization. Iran. J. Pharm. Sci. 3, 61-68.

WAN, L. S., P. W. HENG, L. W. CHAN (1992): Drug encapsulation in alginate microspheres by emulsification. Microencapsul. 9, 309-316.

WAN, L. S., P. W. HENG, L. W. CHAN (1994): Surfactant effects on alginate microspheres. Int. J. Pharm. 103, 267-275.

YAN, W., S. M. FAISAL, S. P. McDONOUGH, T. J. DIVERS, S. C. BARR, C. F. CHANG, M. J. PAN, Y. F. CHANG (2009): Immunogenicity and protective efficacy of recombinant

Leptospira immunoglobulin-like protein B (rLigB) in a hamster challenge model. Microb. Infect. 11, 230-237.

YEH, M. K., Y. T. LIU, J. L. CHEN, C. H. CHIANG (2002): Oral immunogenicity of the inactivated *Vibrio cholerae* whole cell vaccine encapsulated in biodegradable microparticles. Control. Rel. 82, 237-247.

ZHENG, C. H., J. Q. GAO, Y. P. ZHANG, W. Q. LIANG (2004): A protein delivery system: biodegradable alginate-chitosan-poly (lactic-coglycolic acid) composite microspheres. Biochem. Biophy. Res. Commun. 323, 1321-1327.

Received: 19 April 2014
Accepted: 9 May 2015

INANLOU, F., P. KHAKI, N. MOHAMMADPOUR, S. MORADI BIDHENDI, H. ZOLFAGHARIAN, YUNG-FU CHANG, M. R. BAZRAFSHAN, F. SARAEI: Priprava mikrokuglica s antigenom leptospira od biološki razgradivih alginatnih polimera. Vet. arhiv 85, 395-406, 2015.

SAŽETAK

Leptospiroza je zoonoza prouzročena patogenim vrstama roda *Leptospira*, a proširena je diljem svijeta. Iako današnja cjepiva protiv leptospiroze, proizvedena od cijelih bakterijskih stanica, mogu pružiti zaštitu od leptospiroze, potrebna su daljnja istraživanja nove generacije cjepiva koja će moći potaknuti tvorbu dugotrajne imunosti. Biološki razgradive mikrokuglice istražuju se desecima godina kao mogućnost sporog otpuštanja antigena, a posebice su zanimljive one od hidrofilnih polimera kao što je alginat i kitosan, koji imaju izvrsnu biološku kompatibilnost, nisu toksični, a biološki su razgradivi. Svrha ovog rada bila je pripraviti alginatne mikrokuglice i odrediti njihova svojstva pogodna za otpuštanje antigena u postupku imunizacije protiv leptospiroze. Alginatne mikrokuglice s antigenom leptospira bile su pripravljene postupkom emulgacije te im je bio određen oblik, opseg distribucije, učinkovitost ugradnje antigena u mikrokuglice, kapacitet ugradnje i profil otpuštanja antigena. Istraženi su učinci nekih pokazatelja (kao što je koncentracija alginata i emulgatora te omjer miješanja) na obilježja mikrokuglica. Procijenjeni su optimalni uvjeti za pripravu alginatnih mikrokuglica na koje je vezan antigen leptospira. Optimalna koncentracija za alginat bila je 3,5 % (w/v), a emulgator span 80 (0,2 % w/v) i tween 80 (3,75 % w/v). Odgovarajuća homogenizacija postignuta je na 500 okretaja. Rezultati su pokazali da je srednja veličina mikrokuglica bila 200 μm, učinkovitost ugradnje antigena 97 %, a kapacitet 8 %. *In vitro* je ustanovljeno da se antigen leptospira oslobađao s alginatnih mikrokuglica tijekom 192 sata. Ti rezultati pokazuju da alginatne kuglice mogu biti od posebnog interesa za oslobađanje antigena leptospira u organizmu.

Ključne riječi: *Leptospira* spp., antigen, alginat, mikrokuglice, otpuštanje antigena, imunizacija